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Abstract

Respiratory growth and various stress conditions repress RNA polymerase III (Pol III) transcription in Saccharomyces cerevisiae. Here we report a correlation between Pol III transcription repression and degradation of the largest Pol III catalytic subunit, C160. We observed C160 degradation in response to transfer of yeast from fermentation to respiration conditions, as well as treatment with rapamycin or inhibition of nucleotide biosynthesis. We also detected a ubiquitylated form of C160 and demonstrated that C160 protein degradation is dependent on proteasome activity. A comparable study of changes in C160 levels and C160 association with chromatin suggested that modification of C160 that promotes its degradation is not required to release Pol III from tDNA upon transfer of yeast from glucose to respiratory growth conditions. However, the steady state levels of other Pol III subunits besides C160 are also decreased upon this transfer, but the stability of the Pol III complex increased. The results indicate that Pol III repression activates degradation of C160 to reduce the amount of existing Pol III complex and prevent its de novo assembly.

Keywords	RNA polymerase III; yeast; tRNA transcription; protein degradation
Taxonomy	Molecular Mechanism of Gene Regulation, Molecular Biology Experimental Approach

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May 30, 2018

Dear Editor,

Please find enclosed our manuscript entitled "Repression of RNA polymerase III in *Saccharomyces cerevisiae* is correlated with degradation of its largest subunit, C160" which we would like to be considered for publication in BBA Gene Regulatory Mechanisms.

Previous studies have shown a role of ubiquitylation and proteasomal degradation in control of the largest catalytical subunits of both, RNA polymerase I and II. We feel that so far undescribed degradation of the largest subunit of RNA polymerase III is of general interest and our results will be relevant.

Thank you very much for your efforts.

Sincerely,

Magdalena Boguta

Repression of RNA polymerase III in *Saccharomyces cerevisiae* is correlated with degradation of its largest subunit, C160

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Abstract

Respiratory growth and various stress conditions repress RNA polymerase III (Pol III) transcription in *Saccharomyces cerevisiae*. Here we report a correlation between Pol III transcription repression and degradation of the largest Pol III catalytic subunit, C160. We observed C160 degradation in response to transfer of yeast from fermentation to respiration conditions, as well as treatment with rapamycin or inhibition of nucleotide biosynthesis. We also detected a ubiquitylated form of C160 and demonstrated that C160 protein degradation is dependent on proteasome activity. A comparable study of changes in C160 levels and C160 association with chromatin suggested that modification of C160 that promotes its degradation is not required to release Pol III from tDNA upon transfer of yeast from glucose to respiratory growth conditions. However, the steady state levels of other Pol III subunits besides C160 are also decreased upon this transfer, but the stability of the Pol III complex increased. The results indicate that Pol III repression activates degradation of C160 to reduce the amount of existing Pol III complex and prevent its *de novo* assembly.

Introduction

RNA synthesis in the eukaryote nucleus is carried out by the multisubunit RNA polymerases I, II, and III. Whereas Pol I and Pol II synthesize ribosomal and mainly messenger RNA, respectively, Pol III transcribes small RNAs, including transfer RNAs, 5S ribosomal RNA, and U6 small nuclear RNA. Yeast *Saccharomyces cerevisiae* Pol III comprises 17 subunits. The Pol III core, composed of ten subunits, is conserved relative to Pol I and Pol II. The largest catalytic Pol III subunit, C160, shows substantial homology to the largest subunits of Pol I and Pol II, A190 and Rpb1, respectively. Rbp1 contains a repetitive carboxyl terminal domain (CTD), unique to Pol II which, depending on its phosphorylation state, controls subsequent steps of transcription and processing of the primary transcript [1]. Specific features of a carboxyl-terminal extension of C160 that are specific for Pol III were revealed by structure analysis [2] but relevant functional studies are lacking for this subunit.
Over two decades ago Rbp1 was identified as a target for ubiquitylation-mediated degradation in response to DNA damage [3,4]. Blockage of transcription due to DNA damage promotes

Rbp1 ubiquitylation in the nucleus and its proteasomal degradation is associated with gene transcription [5,6]. Ubiquitylation of Rpb1 is reversible in that once DNA damage is repaired, the ubiquitin moiety is removed by the ubiquitin protease Ubp3, which in turn stabilizes Pol II [7]. Pol II proteolysis is an evolutionarily conserved, tightly regulated, multistep pathway [8]. The *S. cerevisiae* Rsp5 protein was the first ubiquitin ligase implicated in Rpb1 degradation and is the only essential HECT family ubiquitin ligase in budding yeast [9]. Pol II ubiquitylation is signed by phosphorylation of CTD, the site of Rsp5 association [10–12]. NEDD4, the mammalian homologue of Rsp5, was also shown to bind to and ubiquitylate Pol II [13]. In addition, polyubiquitylation of Rbp1 for proteasomal degradation requires the Elc1–Cul3 ubiquitin ligase complex that acts in tandem with Rsp5 [14]. Degradation of Rpb1 in the presence of DNA damage requires the Cdc48 protein, a component of the ubiquitin-proteasome system that interacts with the chromatin remodeling complex INO80 that can disrupt contacts between ubiquitylated Rpb1 and chromatin [15]. Although originally identified as a response to DNA damage, Rpb1 degradation also occurs under a number of conditions that lead to Pol II stalling/arrest during transcript elongation [8]. There are at least two alternative pathways that regulate Rpb1 degradation and depend on the stress type. In response to rapamycin, which induces stress similar to that associated with nutrient limitation, chromatin-bound Rpb1 is degraded by a ubiquitin-independent mechanism involving the Rrd1 peptidyl prolyl isomerase [16].

Expression of the largest Pol I subunit, A190, both in yeast and mammals, is also controlled by ubiquitylation and proteasomal degradation [17,18]. In yeast, A190 ubiquitylation serves as a checkpoint for a cold-sensitive step during rRNA transcription. The A190 protein is stabilized via Ubp10-mediated deubiquitylation that is required to achieve optimal levels of ribosomes and cell growth [17]. In contrast to Rpb1, DNA damage and rapamycin treatment has no effect on A190 levels [17,19], indicating that Pol I degradation has a different regulation pathway than does Pol II.

We are interested in mechanisms that control Pol III biogenesis and activity. Pol III is specialized to carry out high-level transcription of short DNA templates and, like Pol I, is regulated in a global manner. Previous studies explored mechanisms that account for repression of Pol III-mediated transcription (referred by [20]). In negative regulation of Pol III transcription in yeast, the Maf1 protein mediates various stress signals to Pol III [21,22]. Under stress conditions, Maf1 directly binds the Pol III complex, which then dissociates from chromatin [23]. Pol III levels are also down-regulated by TOR signaling via phosphorylation of the Pol III subunit C53 [24].

Here we report a decrease in steady state levels of the largest subunit of Pol III, C160, under various conditions that repress Pol III gene transcription. We discovered that the C160 protein is also ubiquitylated and degraded by proteasomes, similar to the largest subunits of Pol I and Pol II. Moreover, we analyzed C160 degradation in the context of association of Pol III with chromatin and stability of the Pol III complex.

Material and Methods

Growth conditions

Yeast were grown in: rich media YPD (2% glucose, 2% peptone, 1% yeast extract), or YPGly (2% glycerol, 2% peptone, 1% yeast extract) or minimal media SC-ura or SC-trp, (2% glucose, 0.67% yeast nitrogen base, supplemented with 20 μg/ml of the amino acids required for growth, except for uracil or tryptophan, respectively). 200 μg/ml geneticin, 200 ng/ml rapamycin (RAP) or 100 μg/ml cycloheximide (CHX) were added to YPD, as required. For nucleotide depletion strains grown on SC-ura with 100 μg/ml mycophenolic acid (MPA) or 100 μg/ml 6-azauracil (6-AU). For testing proteasomal degradation YPGly medium was supplemented with 50 μM MG132 or equal amount of dissolvent – DMSO. Sporulation medium contained 0.05% glucose, 0.1% yeast extract and 1% potassium acetate. Solid media contained 2% agar. All reagents used for media were Difco products.

Yeast strains and plasmids

The yeast strains used in this study are listed in Supplementary Table 1. For testing C160 stability upon nucleotide depletion, MW4415 strain was transformed with empty uracil plasmid – pFL44L [25]. Overexpression of His-tagged ubiquitin was performed from 6His-Ub (*ampR/TRP1*) plasmid [26].

For tagging C160 with TAP or HA in different strains, appropriate sequence was amplified by PCR, using as a template the genomic DNA of MB159-4D derivative encoding C160-TAP or MW4415 strain encoding C160-HA and specific C160TAPF (5'-GGACGCTGTTGAAGGTGTTT-3') and C160TAPR (5'-AAACGTTGACGCGAGTTTTT-3') primers. Transformants were selected on SC-ura for C160-TAP or on YPD medium supplemented with geneticin for C160-HA. In every case, the addition of tag sequence was confirmed by PCR and by western blot method with PAP (Sigma-Aldrich) or HA (Covance) antibody.

The EM4-3C strain was obtained by genetic cross of W303 C160-HA and FA212/10-2D. Deletion of *RRD1* was confirmed by PCR with RRD1-338 (5'-TGAGTCTCGTTCGCCTCTTT-3') and RRD1_307 (5'-TACCAGGCCCCTTCTCTTG-3') primers. Presence of C160-HA was confirmed by PCR using C160TAPF and C160TAPR primers, and by western blotting with HA-specific antibody.

The YPH499 C160-TAP *ump1Δ* strain was obtained by transformation of *ump1Δ* deletion with *TRP1* cassette amplified by PCR, using as a template the genomic DNA of YAS6 strain and primers UMP1_A (5'-CATGTGATGTGACTAGTGTTTGTGA-3') and UMP1_D (5'-TGGGCTGAGAAGTTGAGTATATAGG-3'). Transformants were selected on SC-trp and replacement of *UMP1* locus by *TRP1* deletion cassette was confirmed by PCR.

Protein extraction and western blot analysis

The protein extraction was described earlier [27]. Protein extracts were separated on SDS-PAGE using an acrylamide : bisacrylamide ratio of 37:5:1, for detection of Maf1 phosphorylation the modified acrylamide : bisacrylamide ratio (33.5:0.3) was used. After electrophoresis proteins were transferred to nitrocellulose membrane (Milipore). The membrane was blocked in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) containing

5% fat-free dry milk for 30 min and then incubated with appropriate antibody: mouse monoclonal antibodies anti-HA (Covance) at a 1:5000 dilution for o/n at 4 °C, anti-GFP (Roche) at a 1:5000 dilution for 1 h, anti-Ubiquitine (ENZO) at a 1:1000 dilution for o/n at 4 °C, anti-Vma2 (Molecular Probes) at a 1:10000 dilution for 1 h, anti-Pgk1 (Abcam) at a 1:20000 dilution for 1 h and anti-Actin (Merck-Millipore) at a 1:2000 dilution for 1 h, rabbit polyclonal antibodies anti-HA (Sigma) at a 1:2000 dilution for 1 h, anti-C82 at a 1:5000 dilution for o/n at 4 °C, anti-C53 at a 1:20000 dilution for o/n at 4 °C, anti-AC40 (Gramsh) at a 1:1000 dilution for o/n at 4 °C and anti-Maf1 at a 1:10000 dilution for o/n at 4 °C. Antibodies specific for Pol III subunits (except AC40) and Maf1 specific antibody were a gift from O. Lefebvre. Blots with TAP-tagged proteins were incubated with anti-PAP antibody at a 1:3000 dilution for 1h. PAP is peroxidase anti-peroxidase antibody with no need of secondary antibody. Than membrane was incubated with secondary antibody at a dilution of 1:5000 for 1 h. Secondary anti-mouse or anti-rabbit antibody was coupled to horseradish peroxidase (DAKO). The signal on the membrane was visualized by chemiluminescence using the ECL detection kit (Bio-Rad).

Immunoprecipitation of C160-HA

Yeast cells expressing HA epitope-tagged C160 (MW4415) were grown in YPD medium to exponential phase, than shifted to pre-warmed YPGly medium and incubated for 30 min at 37 °C. From both conditions, pellets, corresponding to 100 ml of culture, were resuspended in 0.5 ml IP buffer (50 mM HEPES-KOH [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.05% NP-40, 0.5 mM DTT, 5% glycerol, O-Complete protease inhibitor [Roche]). Lysis was performed in the presence of glass beads using a Vibramax disruptor (GENE) at maximum speed for 1 h at 4 °C, than beads were washed with 0,3 ml IP buffer. The cell debris were eliminated by centrifugation (20 min at 4°C and 14000 rpm). The protein concentration was determined with the Bio-Rad protein assay. 100 µl of a suspension of Dynabeads PanMouse IgG magnetic beads (Invitrogen), washed three times with 0.5% bovine serum albumin in phosphate-buffered saline (PBS), was incubated with mouse monoclonal anti-HA antibody (6 µg) for 3.5 h at 4 °C. After washing in 0.5% bovine serum albumin in PBS (two times) and then in IP buffer (two times), the beads were incubated overnight with 4 mg of protein extracts in IP buffer with gentle shaking at 4 °C. Then beads were washed three times with IP buffer. Protocol is based on [28]. Immunoprecipitated proteins were released from the beads by boiling them for 5 min and separated by 6% SDS-PAGE and analyzed by western blotting with anti-HA antibody.

Tandem purification of C160-TAP and His-Ub

Yeast cells expressing TAP epitope-tagged C160 and transformed with 6His-Ub plasmid were grown in SC-trp medium at 30 °C. Expression of tagged ubiquitin was induced by $CuSO_4$ addition to final concentration 100 µM, then cells were grown for 4 h to exponential phase. 1.6 l of culture was harvested, washed with cold water and stored at -80°C for further analyzes. Pellet was resuspended in 10 ml TMN150 buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 0,1% NP-40, 1.5 mM $MgCl_2$, 5 mM β-mercaptoethanol) with 2x concentrated O-Complete protease inhibitor (Roche). Lysis was performed in the presence

of zircon beads using a Vortex (GENE) in 5 cycles (1 min of disruption, 1 min incubation on ice). The cell debris were eliminated by centrifugation (30 min at 4 °C and 14000 rpm). Protein extract was incubated with 1 ml IgG Sepharose (SIGMA), washed two times with 5 ml TMN150 buffer and once with 2 ml TMN150 buffer with O-complete for 2 h in 4 °C with rotation. After washing two times with 10 ml TMN1000 buffer (the same as TMN150 but with 1M NaCl) and two times with 5 ml TMN150 buffer, purified proteins were eluted in denaturing conditions two times with 300 µl Ni-WBI buffer (50 mM Tris-HCl [pH 7.8], 300 mM NaCl, 0,1% NP-40, 10 mM imidazole, 1.5 mM $MgCl_2$, 6 M guanidinium chloride, 5 mM β-mercaptoethanol) for 10 min in RT with rotation. Protein eluate was added to 200 µl Ni-NTA agarose, washed two times with 1 ml TMN150 buffer, and incubated 3 h in in 4 °C with rotation. Then Ni-NTA Agarose (Sigma) was washed ones with 1 ml Ni-WBI buffer and two times with 1 ml Ni-WBII buffer (50 mM Tris-HCl [pH 7.8], 50 mM NaCl, 0,1% NP-40, 10 mM imidazole, 5 mM β-mercaptoethanol), proteins were eluted with 300 µl Ni-EB buffer (the same as Ni-WBII, but with 150 mM imidazole) for 5 min in RT with rotation. Eluted proteins were precipitated o/n at -20 °C with 1.5 ml acetone and 1 µl glycoblue, then resuspended in 30 µl 2x SB buffer and boiled 5 min in 98 °C. Proteins were separated by 6% SDS-PAGE and analyzed by western blotting with anti-PAP and anti-Ub antibodies.

AC40-GFP and C160-HA co-immunoprecipitation

Yeast cells expressing HA epitope-tagged C160 and AC40-GFP or expressing only HA epitope-tagged C160 (MW4415) were grown in YPD medium to exponential phase, than shifted to pre-warmed YPGly medium and incubated for 2 hours at 37 °C. Co-immunoprecipitation was performed as described for C160 in [28] except that 0.5 mg of protein extract was used to immunoprecipitation.

Chromatin immunoprecipitation (ChIP)

Yeast cells expressing HA epitope-tagged C160 (MW4415) were grown in YPD medium to exponential phase, than shifted to pre-warmed YPGly medium and incubated for 2 hours at 37 °C, from both conditions 17 OD_{600} units of cells were collected. Yeast chromatin isolation and sonication were prepared as described previously [23]. The preparation of magnetic beads, immunoprecipitation (anti-HA), elution from beads, decrosslinking and DNA purification were done as described previously [29]. For ChIP experiment, the input and immunoprecipitated samples were assayed by quantitative PCR to assess the extent of protein occupancy at different genomic regions. The sequence of primers used are available upon request. PCR reactions contained 2 µl of DNA template, 300 nM primer pairs and 5 µl of 2×SYBR Green reaction mix (ABO). Quantitative PCR was performed on Roche LightCycler 480 using a 5 min soak at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 55 °C and 20 s at 72 °C (with a plate read after each cycle). A melting curve analysis was performed for each sample after PCR amplification to ensure that a single product of expected melting curve characteristics was obtained. Occupancy values (in arbitrary units) were calculated by determining the apparent immunoprecipitation efficiency (the amount of PCR product in the immunoprecipitated sample divided by the amount of PCR product in the input sample).

Results

C160 subunit has limited stability that varies depending on Pol III transcription activity

To evaluate C160 protein stability, yeast cells encoding C160 tagged with the HA epitope were treated with cycloheximide (CHX) in a time-course experiment, and cell lysates were analyzed by immunoblotting. The blots were probed with anti-HA antibodies followed by antibodies directed against other Pol III subunits and the Pol III repressor, Maf1. The level of actin was used to normalize sample loading. The blot showed that incubation with CHX for 120 min resulted in continuing C160 degradation as evidenced by a decrease in C160 levels of relative to untreated cells (Fig. 1A). Meanwhile the levels of Pol III-specific subunits C82 and C53 appeared to be also reduced. After 30 min of CHX treatment, Maf1 was dephosphorylated likely due to stress excreted by CHX, but Maf1 protein levels were not affected as the incubation time increased. This observation is consistent with a mode of Maf1 control by phosphorylation rather than expression levels [30].

Reduced steady state levels of C160 protein were previously detected in the *rpc128-1007* mutant that shows defective Pol III complex assembly [28]. Quantification of the blots showed that incubation of wild type cells with CHX for 120 min resulted in a decrease in C160 levels of about 50% relative to untreated cells (Fig. 1B). In the CHX assay, C160 stability in *rpc128-1007* cells was indeed lower relative to the control strain (Fig. 1B). Since Rpb1 levels are not affected in *rpc128-1007* cells [28], we suspected that this specific degradation of C160 is a consequence of inefficient Pol III-mediated transcription.

Levels of C160 protein decline under stress conditions that repress Pol III transcription

To further explore the correlation between the decrease in C160 levels and Pol III activity, we examined the effect of various conditions known to repress Pol III transcriptional activity. First we used rapamycin, which induces transcriptional effects similar to those seen for nutrient limitation and inhibits Pol III transcription genome-wide [31]. Treatment of wild type cells with rapamycin down-regulated C160 levels by 80% after two hours. However, these C160 levels were higher than that seen in rapamycin-treated *rrd1*Δ mutant cells that are deficient in peptidylprolyl isomerase, which may play a role in C160 degradation (Fig. 2A). Perhaps, in response to rapamycin treatment, the same mechanism used to degrade excess Pol II and Pol III may be induced.

Transcription elongation *in vivo* can be inhibited by 6-azauracil (6-AU), which blocks UTP synthesis and also reduces cellular pools of GTP [32] and mycophenolic acid (MPA) that in turn impairs *de novo* guanosine nucleotide synthesis [33]. Although all RNA polymerases require nucleotides as substrates for RNA synthesis, the effect of nucleotide-depleting drugs is especially pronounced for Pol I and Pol III [34]. In contrast, mRNA synthesis driven by Pol II is only slightly decreased by MPA and 6-AU treatment, with the exception of some transcripts involved in nucleotide biosynthesis that show specific up-regulation [35,36]. Since 6-AU treatment significantly down-regulates tRNA synthesis [34], we assessed how it affects C160

levels. Yeast cells encoding C160-HA were collected at different time points after 6-AU or MPA addition and examined by western blotting with HA-specific antibody (Fig. 2B). Analysis of equal amounts of crude extracts showed a nearly two-fold decline in C160 levels after inhibition of nucleotide synthesis for one hour, and this decline presumably reflected C160 degradation.

Previously we showed a sharp decrease in the levels of newly synthesized Pol III transcripts during transition of yeast from fermentative to glycerol-based medium followed by incubation at elevated temperature [37,38]. Based on these results we examined the effect of this transition on C160 expression. Cells encoding C160-HA fusion that were grown in glucose medium under optimal conditions (YPD) were transferred to medium containing glycerol (YPGly) and incubated at 37 °C. Western blot analysis of samples taken every 30 min showed a continuous decrease of C160 levels to about 40% of initial values within 120 min (Fig. 2C). Notably, transition of yeast from fermentative to glycerol-based growth resulted in decreased levels of C160 proteins fused to various tags and in strains having different genetic backgrounds (Fig. S1). Taken together, these results show that diverse conditions that repress Pol III-mediated transcription lead also to a down-regulation of the largest Pol III subunit, C160.

The switch between fermentation and respiration results in proteasomal degradation of C160

The decrease in C160 protein levels under conditions that cause Pol III repression suggested that C160 expression could be controlled by the ubiquitin proteasome system. Ubiquitylation of yeast C160 protein has been detected in three independent proteome-wide screens [39–41], but has not been validated by molecular studies.

To identify ubiquitylated C160 forms, HA-tagged C160 was immuno-purified from yeast grown first in glucose medium and then shifted to medium containing glycerol with incubation at 37 °C for 30 min (Fig. 3A). Analysis of immuno-purified fractions by western blotting with HA-specific antibody identified additional bands that migrated slower than the predicted 170 kDa C160-HA protein, suggestive of C160 ubiquitylation (Fig. 3A).

To examine C160 ubiquitylation, a two-step purification involving TAP-tagged C160 and His-tagged ubiquitin was performed. Incubation of crude cell extracts with IgG Sepharose that can bind the TAP epitope resulted in a near complete depletion of C160 from the supernatant (Fig. 3B, left panel). The immuno-purified C160-TAP was then incubated with nickel resin following by western blot analysis of the eluted fraction with TAP- and ubiquitin (Ub)-specific antibodies. Both assays identified a single band migrating above 170 kDa (Fig. 3B, right panel) that was predicted to correspond to the ubiquitylated form of C160-TAP.

To determine if C160 levels were post-translationally regulated by a proteasome-mediated degradation pathway, we assessed the effects of the proteasome inhibitor MG132 on C160 protein levels. Because wild-type yeast contain transport proteins that actively pump MG132 out of cells, we analyzed cells lacking the major drug transporters Pdr5 and Snq2. Yeast were grown in glucose medium and transferred to medium with glycerol followed by a 2 h incubation at 37 °C. Treatment of cells grown in glycerol medium with the proteasome

inhibitor MG132 showed increased endogenous C160 protein levels (Fig. 4A, left panel), supporting proteasome-dependent degradation of C160. Moreover, inactivation of the gene encoding the Ump1 protein that is required for 20S proteasome maturation also resulted in increased C160 levels (Fig. 4A right panel).

Ubiquitylation and degradation of the Pol II Rbp1 subunit induced by DNA damage is dependent on the Rsp5 ubiquitin ligase [9,14]. Rpb1 degradation was not observed in temperature-sensitive *rsp5-1* that carries a mutation in the HECT domain of Rsp5 that directly affects its catalytic activity, even when a DNA-damaging reagent was applied at elevated temperatures [42]. Here we examined whether Rsp5 might similarly be involved in ubiquitylation and degradation of C160 upon transfer of yeast to medium with glycerol following 2 h incubation at 37 °C. We used *rsp5-1* and another temperature-sensitive mutant, *rsp5-19*, harboring a substitution in the WW3 domain that governs the interaction of Rsp5 with its substrates [43]. The *rsp5* mutants showed no difference in C160 protein degradation in medium with a non-fermentable carbon source (Fig. 4B) suggesting that mechanisms that regulate C160 ubiquitiylation and degradation differ from those for Rpb1. The levels and phosphorylation status of C53, another Pol III subunit, and Maf1 regulatory protein were also unaffected by *rsp5* alleles (Fig. 4B).

C160 loss is related to dissociation of Pol III from tDNA and enhancement of interactions between Pol III complex subunits

The switch between fermentation and aerobic respiration induced by growth on non-fermentable carbon sources causes widespread changes in gene transcription, including repression of tDNA transcription [37]. Transfer of cells from glucose medium (YPD) to medium with glycerol (YPGly) followed by a 2 h incubation at 37 °C corresponded to a marked decrease in Pol III activity due to strong repression mediated by Maf1 [37]. A significant decrease in levels of primary transcripts of selected tRNA genes is also seen under these conditions [38].

Here, we examined cell growth following a switch from glucose to glycerol-based medium and growth at 37 °C on C160 occupancy of tDNA. Transfer of wild type cells from glucose medium (YPD) to repressive conditions of YPGly decreased C160 occupancy of tDNA-Leu and tDNA-Phe by 81% and 91%, respectively, but during the same interval only about 60% of C160 was degraded (compare Fig. 5A and 5B). These estimates suggested that a substantial portion of the Pol III complex remained intact and was released from chromatin independently of C160 degradation. As determined by western blotting, incubation in YPGly decreased expression of other Pol III complex subunits, although to a lesser extent than for C160 (Fig. 5A).

To determine whether dissociation of the polymerase from DNA leads to disassembly of the Pol III complex, we tested how the transfer of cells from glucose medium to medium with glycerol followed by 2 h incubation at 37 °C affected the association between Pol III components. Using a GFP-specific antibody, the entire complex was immuno-purified from cells expressing AC40-GFP and C160-HA, and the interactions between different Pol III

subunits were examined by co-immunoprecipitation (Fig. 6A). AC40 protein purified from YPD and YPGly cells together with co-purified proteins were subjected to quantitative western blot analysis. The association of AC40 with C82 was clearly enhanced, more than two-fold, by the change in media, but AC40-C160- and AC40-C53 associations were also increased in YPGly cells (Fig. 6A). Moreover, immuno-purification of C160-HA, followed by western blotting also showed an increase in co-immunoprecipitated subunits C82, C53 and AC40 (Fig. 6B).Theses results indicate that Pol III release from chromatin is not related to Pol III complex dissociation, but that interactions between subunits may be even strengthened.

Discussion

In this work we present evidence that inhibition of Pol III transcription in yeast is correlated with degradation of the Pol III catalytic subunit C160. Furthermore, we showed for the first time that C160 is ubiquitylated and degraded by proteasomes. C160 degradation during stress caused by transfer to media that promotes fermentation from media that favors respiration is preceded by Pol III release from chromatin. Although the steady-state levels of other Pol III subunits also decreased, the strength of the Pol III complex association increased in repression conditions.

We sought to address whether, similar to the largest Pol I and Pol II subunits, Pol III regulation involves controlled degradation of the largest subunit of the complex, C160, under conditions that repress Pol III activity. To do this, we examined the kinetics of decreases in steady-state levels of the C160 protein in cells treated with rapamycin, 6-AU or MPA or transferred to medium having a non-fermentable carbon source. The half-life of the C160 protein, determined in the CHX chase experiment, was about 120 min (Fig. 1) and under stress the C160 levels were reduced by half faster, even when *de novo* synthesis was not blocked by CHX (Fig. 2).

Our results strongly support the hypothesis that decreased amounts of C160 arise from protein degradation. First, the effect of rapamycin on C160 expression was nearly identical to that seen for Rpb1, which, in response to rapamycin treatment, is degraded in the chromatin-bound state [16]. The mechanism of C160 degradation in rapamycin-treated cells could be analogous because it is also dependent on the peptidyl prolyl isomerase Rrd1 (Fig. 2). Second, GTP depletion only slightly reduces Pol II-dependent mRNA synthesis except for some transcripts involved in nucleotide biosynthesis that show specific up-regulation [35,36]. Finally, down-regulation of C160 upon transfer of yeast to a non-fermentable carbon source is dependent on proteasome activity, demonstrating that C160 is primarily degraded by the proteasome. Based on our findings we conclude that Pol III inhibition activates degradation of its largest subunit, C160. Interestingly, recently reported data showing destabilization of Pol I machinery in human cells treated with the DNA intercalator BMH-21 support the possibility that inhibition of Pol I activity drives degradation of its largest subunit [44].

Pol II and Pol I are degraded by different mechanisms depending on the type of stress, which may also affect Pol III degradation. Our study focused on C160 degradation and Pol III repression in response to growth on a non-fermentable carbon source. In the natural environment, yeast responds to variations glucose availability by undergoing frequent physiological transitions between fermentation and respiratory growth using non-fermentable

carbon sources. Yeast must also adapt to temperature variations. Here we induced similar metabolic changes by transferring yeast from a glucose-rich medium to a non-fermentable glycerol medium at elevated temperature to monitor C160 expression in the context of Pol III association with chromatin and stability of the Pol III complex.

Our data support and expand the basis for the coupling of carbon metabolism to Pol III transcription. We showed that the catalytic Pol III subunit C160 undergoes proteasomal degradation in response to a shift from a glucose to a glycerol carbon source. Moreover, we detected ubiquitylated C160. Together these findings strongly support a role for ubiquitylation in proteasome-mediated Pol III degradation. Future studies will be needed to define the mechanism that mediates this ubiquitylation, although these findings indicate that Pol III degradation occurs independently of the ubiquitin ligase Rsp5, suggesting that the mechanism differs from that described for Pol II inhibition following DNA damage [8].

We have shown that most Pol III complexes are released from chromatin upon shifting the cells to a non-fermentable carbon source (Fig. 5B). This finding is consistent with our previous work showing a significant decrease in Pol III transcription under similar conditions [37,38]. The relatively smaller decrease in the amount of C160 indicates that designation of C160 for degradation does not necessarily correspond with Pol III complex removal from DNA under repression induced by a non-fermentable carbon source. In contrast, degradation of the yeast Pol II subunit Rpb1 in response to unsuccessful resolution of Pol II blockage at DNA lesions is a rationale for removal of Pol II from DNA and is required for cell survival [8].

C160 is present in excess relative to other Pol III subunits [45], which may be because, as the largest core subunit, C160 serves as a scaffold for Pol III assembly. Under stress conditions, this excess may be unnecessary and thus C160 is degraded. Indeed, general responses to stress often induce proteolysis of unneeded proteins that may be harmful to the cell [46].

Another observation is enhanced interactions between Pol III subunits under repression conditions promoted release of the Pol III complex from chromatin (Fig. 6). Degradation of excess C160 may serve to prevent *de novo* assembly of the Pol III complex under repression conditions but allow recruitment of existing complexes when environmental conditions are favorable for transcription. Overall, these findings suggest that repression of Pol III transcription is associated with Pol III assembly.

CONCLUSIONS

- Repression of RNA polymerase III is correlated with degradation of its subunit, C160
- C160 protein is ubiquitylated and degraded in proteasome
- Interaction between RNA polymerase III subunits is enhanced under repression

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FIGURE LEGENDS

FIGURE 1. (A) The largest protein subunit of Pol III, C160, has limited stability. A yeast strain expressing C160 fused to an HA tag was grown in rich glucose medium until the exponential growth phase was reached and cycloheximide (CHX) was added to a final concentration 100 μg/ml and growth was allowed to proceed at 30 °C. The cells were then harvested as indicated and examined by western blotting using anti-HA antibody and antibodies specific for selected Pol III subunits and Maf1. Actin was used as a loading control. (B). Stability of C160 is decreased in a mutant yeast strain with low Pol III activity. Control (wt) and isogenic *rpc128-1007* cells encoding C160 fused to the TAP epitope were treated with CHX as described above. C160 stability was monitored by western blotting using peroxidase anti-peroxidase (PAP) antibody. C160-TAP levels were normalized to the loading control (Pgk1) and calculated relative to the amount in untreated wild type yeast that was set to 1. Band intensities from western blot images were quantified using MultiGauge v3.0 software (Fujifilm). Bars represent the mean ± standard deviation of three independent experiments. ** and * asterisks indicate, respectively, p value < 0.005 and < 0.05.

FIGURE 2. Reduced levels of C160 protein under conditions that lead to global repression of Pol III-mediated transcription. Strains encoding C160 with an HA epitope were grown in rich glucose medium (YPD) (A and C) or in minimal medium without uracil (B) to the exponential growth phase and then subjected to stress conditions before harvest at the indicated time points: (A) Control (wt) and isogenic *rrd1Δ* cells incubated with 200 ng/ml rapamycin (RAP) at 30 °C. (B) Cells incubated with 100 μg/ml 6-AU or 100 μg/ml MPA at 30 °C; (C) Cells transferred to rich glycerol medium (YPGly) and incubated at 37 °C. C160 levels were monitored by western blotting with anti-HA antibody and quantified as described in Figure 1. Vma2, Pgk1 or actin were used as loading controls. Bars represent the mean ± standard deviation of three independent experiments. ** and * asterisks indicate, respectively, p value < 0.001 and < 0.05.

FIGURE 3. Identification of C160-ubiquitin conjugates. (A) Yeast expressing C160 with HA epitope were grown in rich glucose medium at 30 °C to exponential growth phase (YPD), transferred to rich glycerol medium (YPGly), incubated 30 min at 37 °C and harvested. Immunoprecipitation of C160 was monitored by western blotting with anti-HA antibody (α-HA). TOT, 2% of total protein extracts; FT, protein unbound to α-HA, IP- α-HA protein, bound to α-HA. The middle lane was loaded with a molecular weight marker indicating the position of the 170 kDa protein. The dashed line indicates where the image was cropped to remove irrelevant lanes. (B) Yeast expressing TAP-tagged C160 and His-tagged ubiquitin, grown on minimal medium without tryptophan, were subjected to double immuno-purification. C160-TAP bound by IgG Sepharose resin (S-beads) was eluted and incubated with nickel resin (Ni-beads). Respective fractions were analyzed by western blotting with TAP- or ubiquitin (Ub)-specific antibodies. The same experiment was performed with a yeast strain encoding untagged C160.

FIGURE 4. Proteasome-mediated C160 degradation. Wild type strain, *snq2Δpdr5Δ* and *ump1Δ* mutants (A) or *rsp5* alleles (B), all expressing C160-HA, were grown in rich glucose

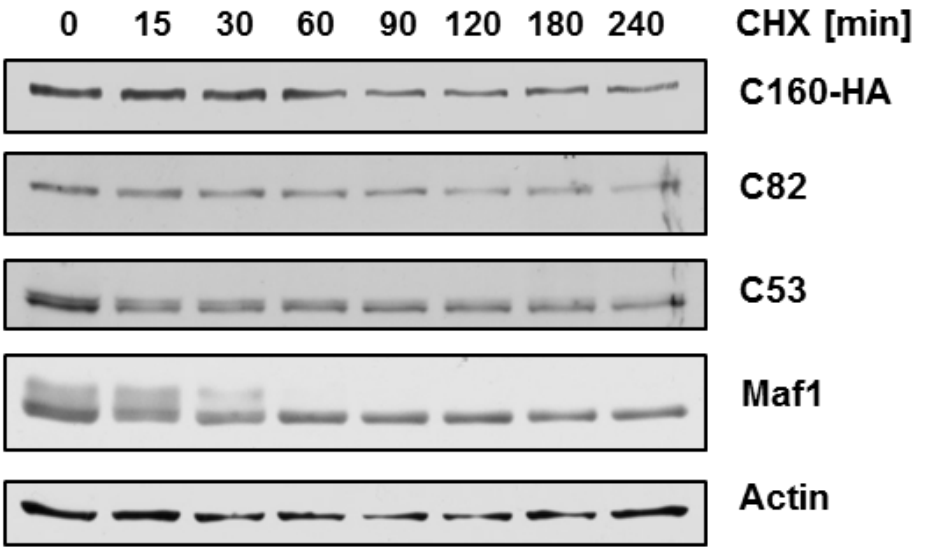
medium (YPD) at the indicated temperatures ((A, left panel with *snq2Δpdr5Δ* strain) 30 °C (A, right panel with *ump1Δ* strain and B) 23 °C), transferred to rich glycerol medium (YPGly) and incubated at 37 °C for 2 h. Upon transfer to YPGly, *snq2Δpdr5Δ* cells were additionally treated with either DMSO or MG132. Cell lysates were analyzed by western blotting. Vma2 or actin were used as a loading controls. The amounts of C160 protein expressed were quantified as described for Figure 1.

FIGURE 5. Pol III is affected by a switch from fermentation to aerobic respiration. Strain expressing C160-HA and control untagged strain grown in glucose medium were harvested in the exponential phase (YPD) or transferred to glycerol medium and cultivated in YPGly for 2 h at 37 °C. (A) Levels of Pol III subunits were examined by western blotting and quantified as described for Figure 1. Pgk1 was used as the loading control. (B) C160 occupancy on tDNA was determined by ChIP. Cross-linked chromatin was immunoprecipitated with antibodies against HA followed by quantitative real-time PCR with primers specific to single tDNA genes: tL(CAA)G1 and tF(GAA)H1 (designated as tDNA-Leu and tDNA-Phe) The occupancy of C160-HA is represented relative to the level of C160-HA in the wild type strain grown on glucose, which was set to 1. Bars represent the mean ± standard deviation of three independent experiments.

FIGURE 6. Effect of switch of yeast between fermentation and aerobic respiration on interactions between Pol III subunits. Extracts were prepared from cells encoding AC40-GFP and C160-HA (A) or C160-HA (B). Pol III was immunoprecipitated from yeast extracts by GFP-specific (A) or HA-specific antibody (B). AC40 (A) or C160 (B) immune-purified from equal amounts of YPD and YPGly cells were examined by western blotting together with co-purified proteins with antibodies specific for HA, C82, C53 or AC40. The ratio of co-purified and input proteins was calculated. Bars represent the mean ± standard deviation of three independent experiments.

Fig. 1

A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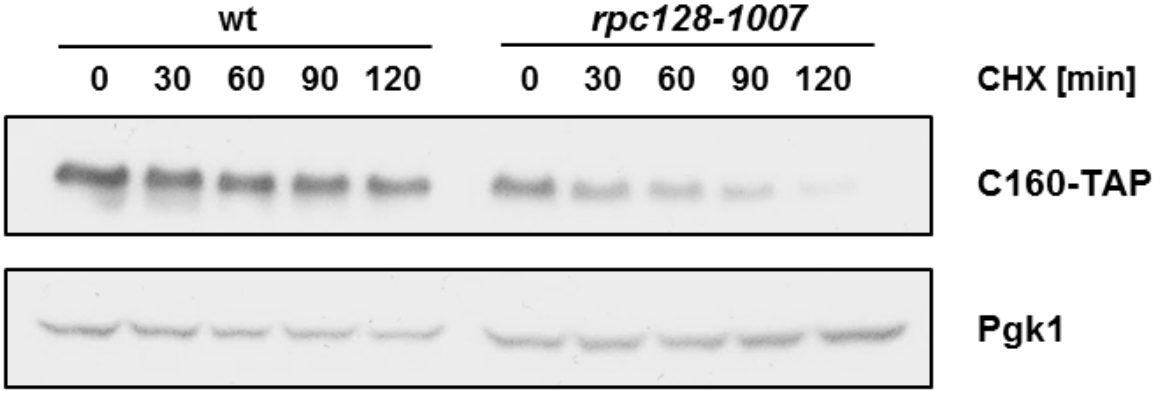
CHX [min]
C160-HA
C82
C53
Maf1
Actin

B


wt
rpc128-1007
0 30 60 90 120
0 30 60 90 120
CHX [min]
C160-TAP
Pgk1

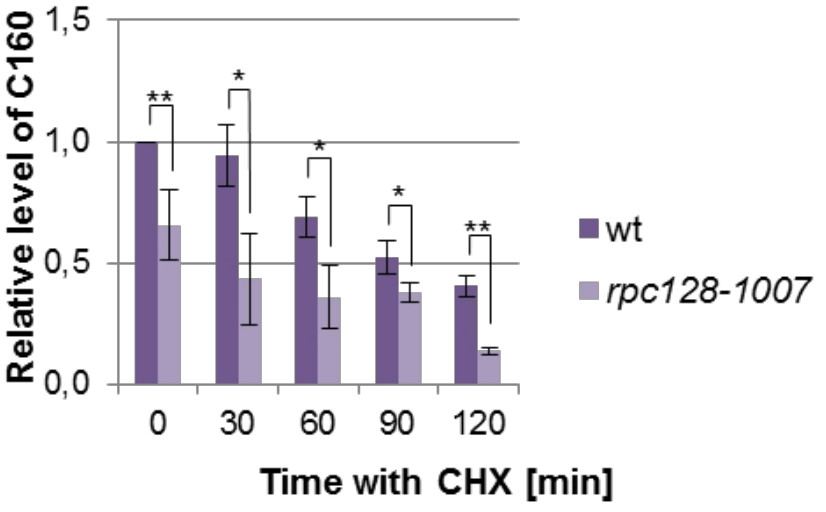

Relative level of C160
1,5
1,0
0,5
0,0
**
*
*
*
**
0 30 60 90 120
Time with CHX [min]
wt
rpc128-1007

Fig. 2

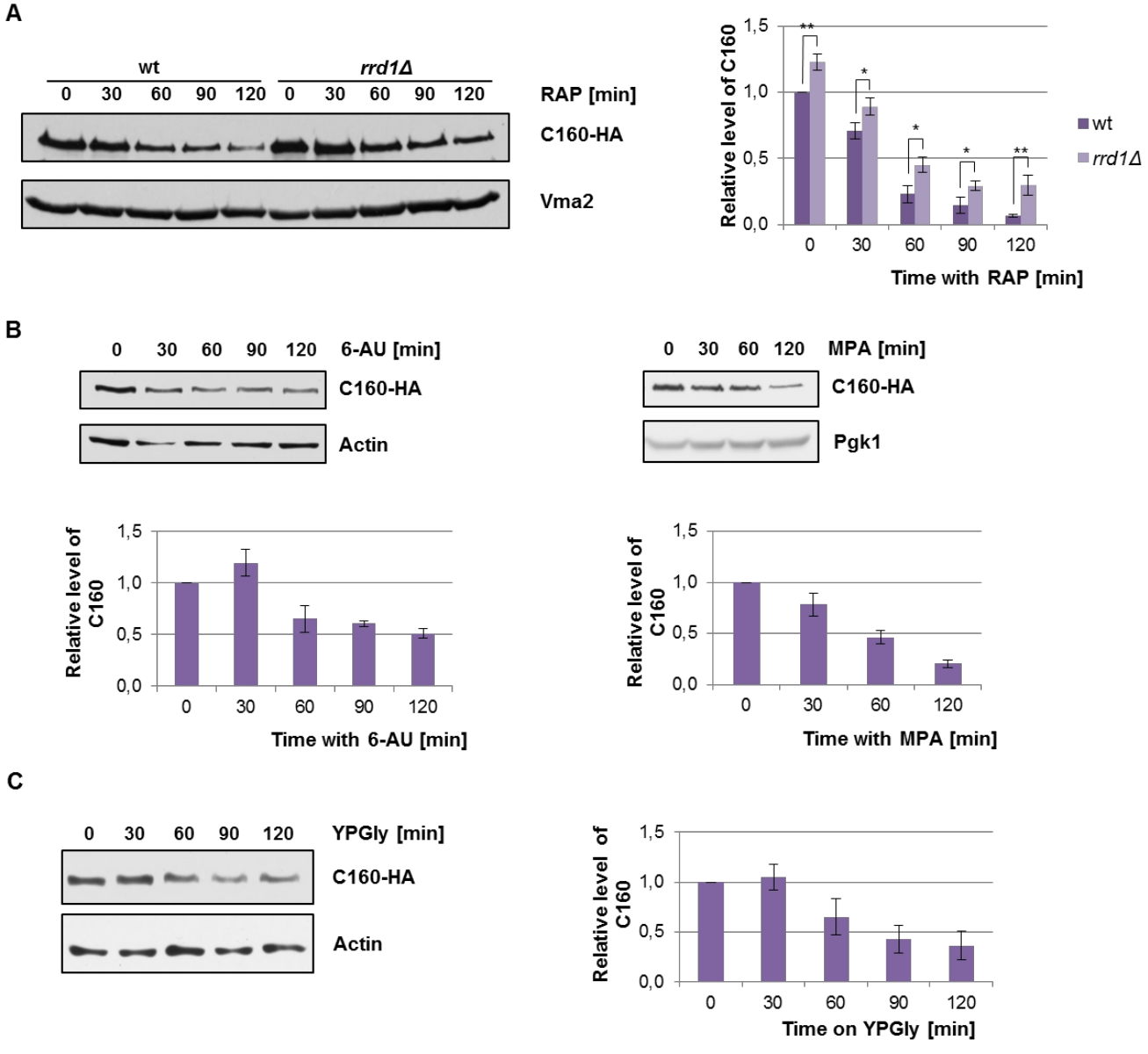

A
wt
rrd1Δ
0 30 60 90 120 0 30 60 90 120
RAP [min]
C160-HA
Vma2
Relative level of C160
1,5
1,0
0,5
0,0
**
*
*
*
**
0 30 60 90 120
Time with RAP [min]
wt
rrd1Δ
B
0 30 60 90 120 6-AU [min]
C160-HA
Actin
Relative level of C160
Time with 6-AU [min]
0 30 60 120 MPA [min]
C160-HA
Pgk1
Relative level of C160
Time with MPA [min]
C
0 30 60 90 120 YPGly [min]
C160-HA
Actin
Relative level of C160
Time on YPGly [min]

Fig. 3

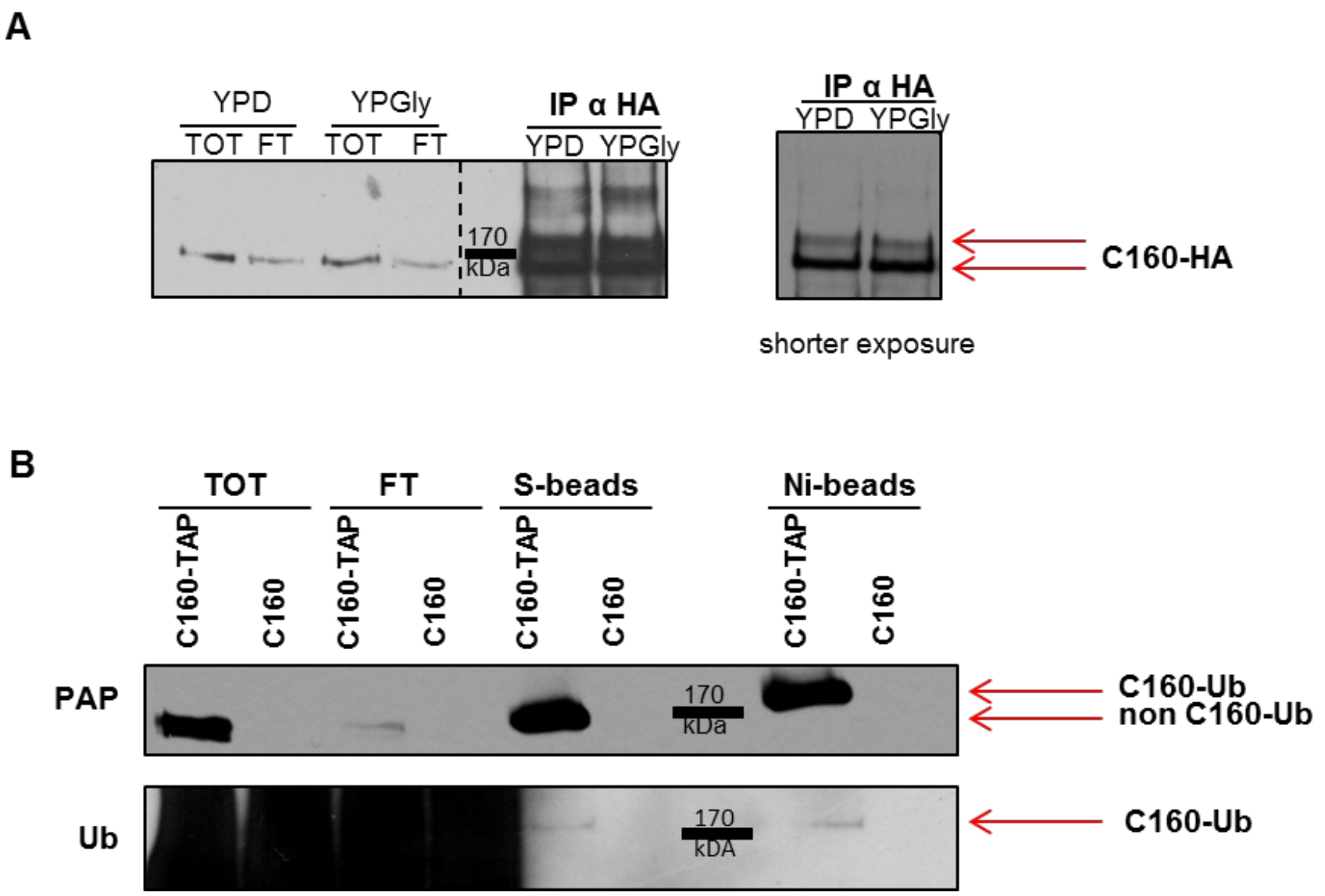

A
YPD
YPGly
TOT FT
TOT FT
IP α HA
YPD YPGly
170
kDa
IP α HA
YPD YPGly
C160-HA
shorter exposure
B
TOT
FT
S-beads
Ni-beads
C160-TAP
C160
C160-TAP
C160
C160-TAP
C160
C160-TAP
C160
PAP
170
kDa
C160-Ub
non C160-Ub
Ub
170
kDA
C160-Ub

Fig. 4

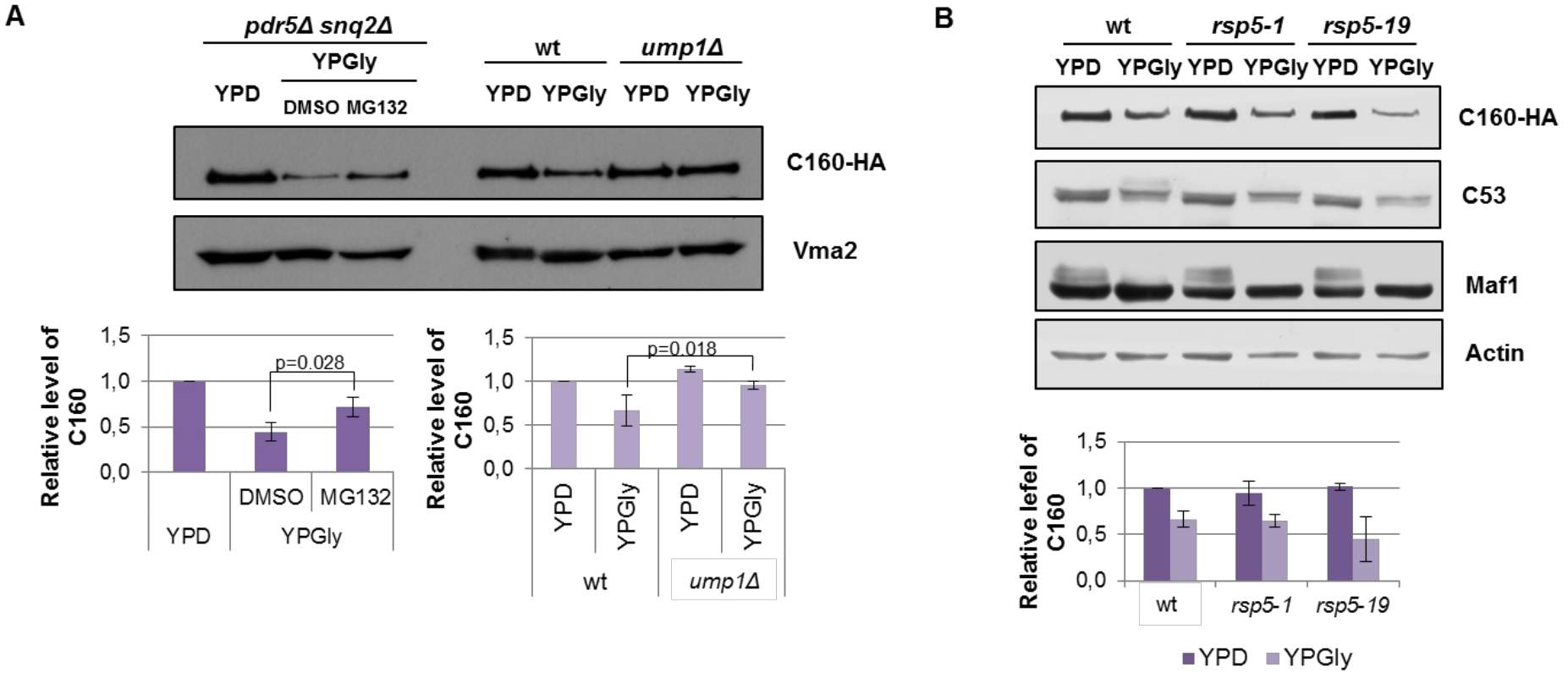
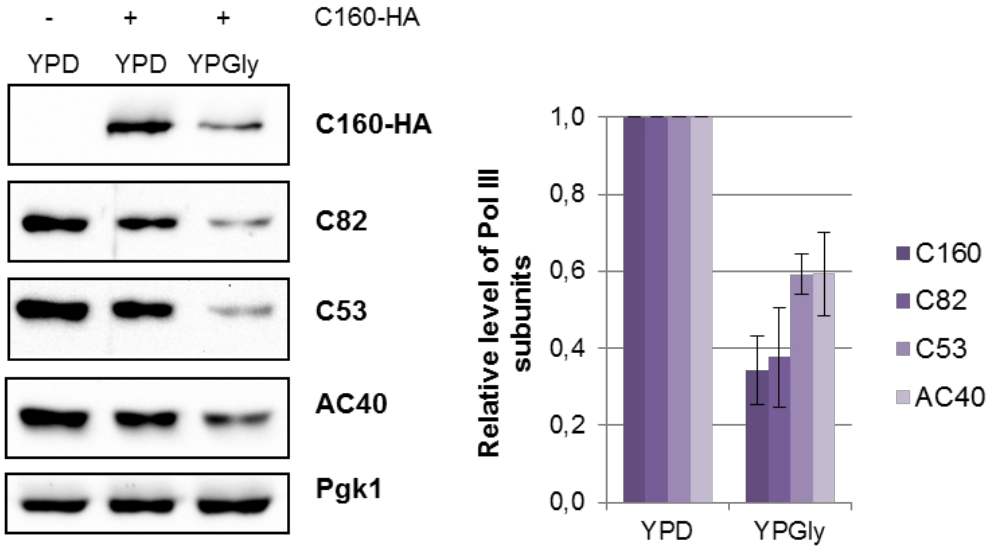

A
pdr5Δ snq2Δ
YPD
YPGly
DMSO MG132
wt
ump1Δ
YPD YPGly YPD YPGly
C160-HA
Vma2
Relative level of C160
1,5
1,0
0,5
0,0
p=0.028
DMSO MG132
YPD
YPGly
p=0.018
wt
ump1Δ
B
wt
rsp5-1
rsp5-19
YPD YPGly YPD YPGly YPD YPGly
C160-HA
C53
Maf1
Actin
Relative lefel of C160
wt
rsp5-1
rsp5-19
YPD YPGly

Fig. 5

A


- + + C160-HA
YPD YPD YPGly
C160-HA
C82
C53
AC40
Pgk1
Relative level of Pol III subunits
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0,8
0,6
0,4
0,2
0,0
YPD
YPGly
C160
C82
C53
AC40

B

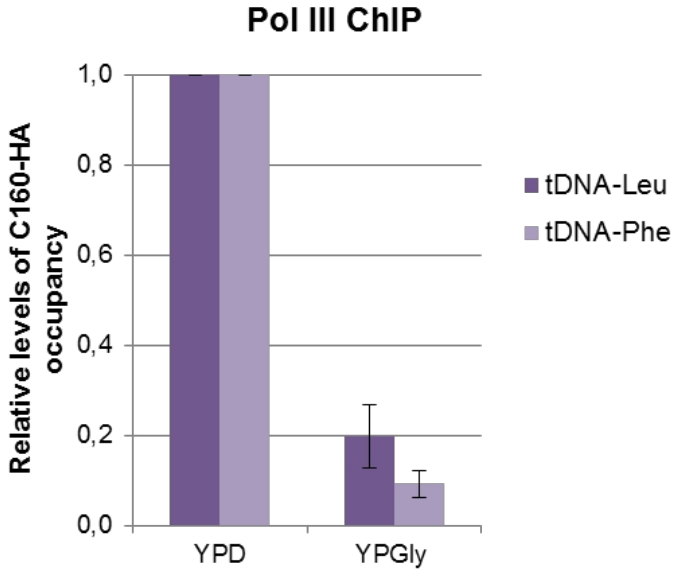

Pol III ChIP
Relative levels of C160-HA occupancy
1,0
0,8
0,6
0,4
0,2
0,0
YPD
YPGly
tDNA-Leu
tDNA-Phe

Fig. 6

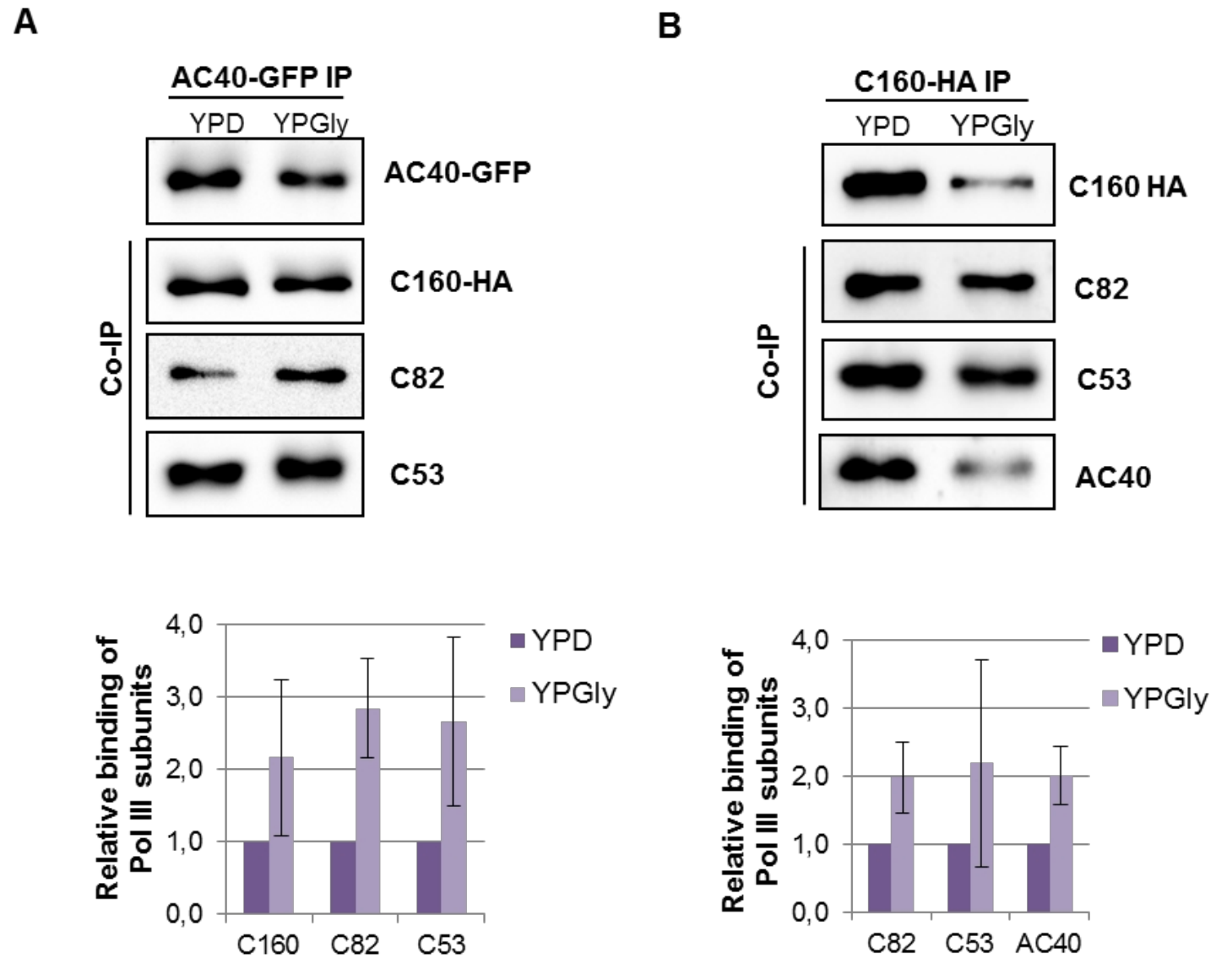
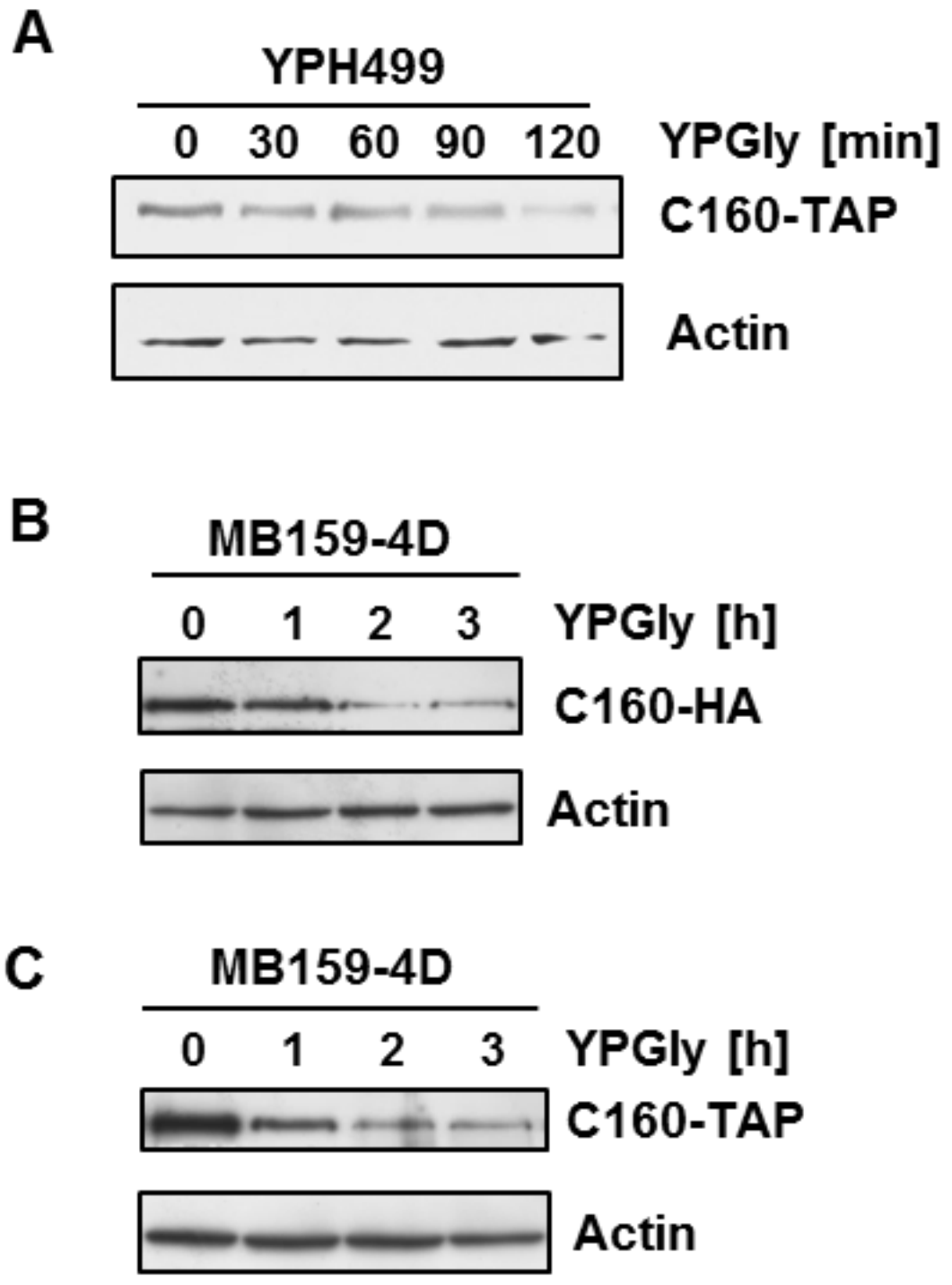

A
AC40-GFP IP
YPD
YPGly
AC40-GFP
C160-HA
C82
C53
Co-IP
Relative binding of Pol III subunits
4,0
3,0
2,0
1,0
0,0
YPD
YPGly
C160
C82
C53
B
C160-HA IP
YPD
YPGly
C160 HA
C82
C53
AC40
Co-IP
Relative binding of Pol III subunits
4,0
3,0
2,0
1,0
0,0
YPD
YPGly
C82
C53
AC40

Fig. S1


A
YPH499
0 30 60 90 120
YPGly [min]
C160-TAP
Actin
B
MB159-4D
0 1 2 3
YPGly [h]
C160-HA
Actin
C
MB159-4D
0 1 2 3
YPGly [h]
C160-TAP
Actin

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5.

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FIGURE S1. Transition of yeast from fermentative to glycerol medium caused down-regulation of C160-HA or C160-TAP in strains having various genetic backgrounds. Derivatives of YPH499 encoding C160 with TAP epitope (A) or MB159-4D encoding C160 with HA (B) or TAP epitope (C) were grown in rich glucose medium (YPD) to exponential growth phase, transferred to rich glycerol medium (YPGly), incubated at 37 °C and harvested at the indicated time points. C160 levels were monitored by western blotting with anti-HA antibody or peroxidase anti-peroxidase (PAP) antibody. Actin was used as a loading control.

Supplementary Table 1. The yeast strains used in this study.

Strain	Genotype	Source of reference	Figure
YPH499	*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63*	[1]	
MB159-4D	*MATa SUP11 ade2-1 lys2-1 leu2-3,112 his3 ura3-1*	[2]	
MJ15-9C	*MATa SUP11 ade2-1 lys2-1 leu2-3,112 his3 ura3-1 rpc128-1007*	[2]	
MW4415	*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63 RPC160::3HA::KanMX6*	[3]	1A, 2B, 2C, 3A, 4A, 4B, 5A, 5B, 6B
YPH499 C160-TAP	*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63 RPC160::TAP-K.l. URA3*	This study	3B, S1A
MB159-4D C160-HA	*MATa SUP11 ade2-1 lys2-1 leu2-3,112 his3 ura3-1 RPC160::3HA::KanMX6*	[4]	S1B
MB159-4D C160-TAP	*MATa SUP11 ade2-1 lys2-1 leu2-3,112 his3 ura3-1 RPC160::TAP-K.l. URA3*	This study	1B, S1C
MJ15-9C C160-TAP	*MATa SUP11 ade2-1 lys2-1 leu2-3,112 his3 ura3-1 rpc128-1007 RPC160::TAP-K.l. URA3*	This study	1B
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	[5]	
W303 C160-HA	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 RPC160::3HA::KanMX*	This study	2A
FA212/10-2D	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 rrd1Δ::KanMX*	[6]	
EM4-3C	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 RPC160::3HA::KanMX rrd1Δ::KanMX*	This study, genetic cross of W303 C160-HA and FA212/10-2D	2A
YAS6	*MATα can1-100 ade2-1 lys2-1 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ900 GAL⁺ RAD5 ump1Δ::TRP1*	[7]	

YPH499 C160-HA *ump1Δ*	*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63 RPC160::3HA::KanMX6 ump1Δ::TRP1*	This study	4A
YYM4	*MATa ura3 his3 leu2 trp1 lys2 ade2 pdr5Δ::TRP1 snq2Δ::hisG*	[8]	
YYM4 C160-HA	*MATa ura3 his3 leu2 trp1 lys2 ade2 pdr5Δ::TRP1 snq2Δ::hisG RPC160::3HA::KanMX6*	This study	4A
PC10	*MATα his3Δ-200 leu2-3, 112 ura3-52 lys2-801 trp1-1 rsp5-1*	[9]	
PC7	*MATα his3Δ-200 leu2-3, 112 ura3-52 lys2-801 trp1-1 rsp5-19*	[10]	
PC10 C160-HA	*MATα his3Δ-200 leu2-3, 112 ura3-52 lys2-801 trp1-1 rsp5-1 RPC160::3HA::KanMX6*	This study	4B
PC7 C160-HA	*MATα his3Δ-200 leu2-3, 112 ura3-52 lys2-801 trp1-1 rsp5-19 RPC160::3HA::KanMX6*	This study	4B
AC40-GFP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPC40::GFP*	[11]	
AC40-GFP C160-HA	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPC40::GFP RPC160::3HA::KanMX6*	This study	6A

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